Concise Review

A Family of Ligands for the TNF Receptor Superfamily

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Key Words. CD40 • CD30 • CD27 • Fas • OX40 • 4-1BB • Lymphotoxin β • Hyper IgM syndrome

Abstract. Recent progress in the definition of molecules involved in immune regulation has led to the discovery of a number of type I membrane glycoproteins with a distinctive, cysteine-rich, repetitive domain structure within their extracellular regions. Because the prototype members of this family are receptors for cytokines (tumor necrosis factor [TNF] and nerve growth factor [NGF]), it was expected that the ligands for the other receptors would possess cytokine-like activities. This prediction has been fulfilled by the cloning of cDNA encoding a series of type II membrane glycoproteins, with homology to TNF, that bind to, and signal through, their cognate receptors. While the biological role of some of these ligand-receptor pairs remains obscure, at least two members of the family, CD40 and Fas, have proven their importance. The human X-linked immunodeficiency, hyper IgM syndrome, is the result of mutations in the CD40 ligand gene, and the Fas and Fas ligand genes are mutated in two mouse strains, *lpr* and *gld*, that develop autoimmune disease. These findings, together with other evidence, point to key roles of CD40/CD40 ligand interactions in immune activation, particularly in T-dependent B cell responses, and of Fas/Fas ligand in apoptosis and peripheral tolerance. These molecules, as well as the other ligands of the family, share the property of costimulation of T cell proliferation and are all expressed by activated T cells. More detailed analysis of the expression patterns of ligands and receptors on lymphocyte subpopulations will be necessary to define their different roles in immune activation and suppression.

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Introduction.

The purpose of this review is to summarize current knowledge of the structures and biological activities of an emerging family of ligands and receptors that are structurally related to tumor necrosis factor (TNF) and its receptors. Because TNF has been studied extensively for a number of years and comprehensively reviewed [1, 2], I will concentrate on the more recently characterized members of the family and refer to the TNF system for comparison. Although the low affinity nerve growth factor receptor (NGFR) is related to the TNF receptor (TNFR), nerve growth factor (NGF) and its homologs, the neurotrophins, are not structurally related to TNF and will not be considered here [3, 4].

The Receptor Family

The first of the receptors to be cloned was the low affinity receptor for NGF [5]. The two receptors for TNF, p75 and p55, were also recognized and cloned by virtue of their ability to bind TNF [6-8]. Many of the other family members were first identified by the generation of monoclonal antibodies recognizing the particular cell-surface protein. These antibodies were found to mediate functional effects when cross-linked and/or to stain interesting cell populations, as discussed later.

Additionally, a number of members of the poxvirus family have been shown to encode secreted TNF-binding proteins with strong homology to the extracellular domain of the TNF receptor [9, 10]. Presumably, the viruses acquire these genes from the cellular genome during evolution and use the soluble TNF-binding proteins to antagonize the anti-viral action of TNF during infection of the host.

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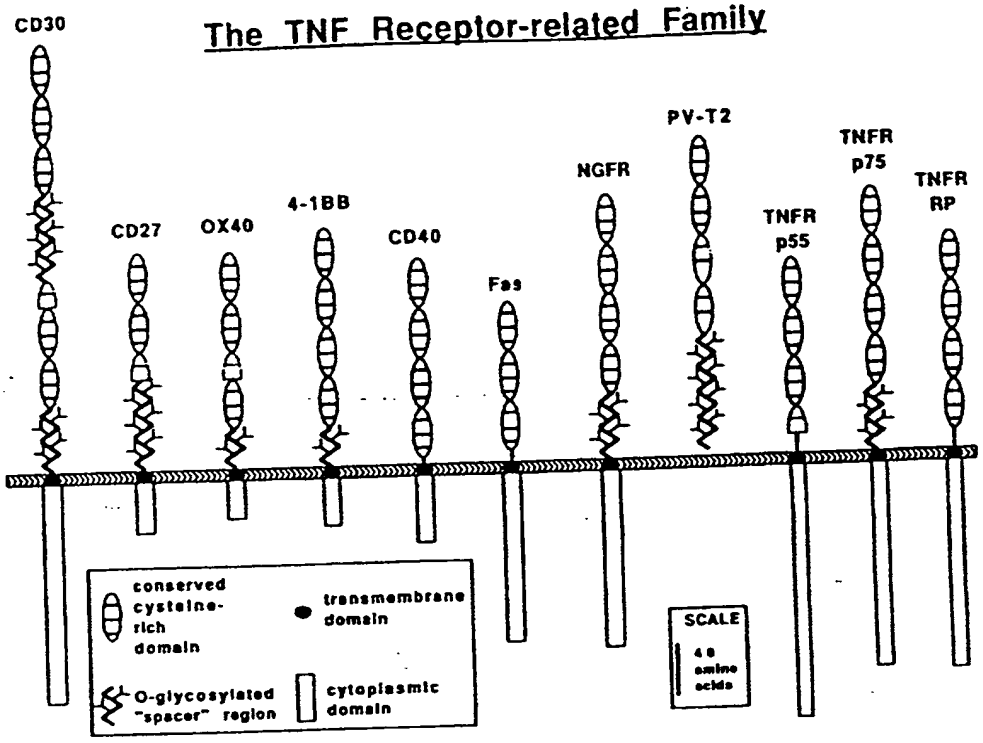

Fig. 1. Schematic representation of the structures of the TNF-receptor-related family. The indicated features of this family of type I membrane glycoproteins include the repeated, conserved, cysteine-rich domains of about 40 amino acids, and the O-glycosylated "spacer" region. Note that PV-T2 is a secreted, pox-virus encoded, TNF-binding protein.

With the exception of these virally encoded proteins, the remaining family members (CD40 [11], CD30 [12], CD27 [13], 4-1BB [14], Fas [15], OX40 [16] and TNFR-related protein (TNFR-RP) [17]) are type I membrane glycoproteins characterized by the presence of variable numbers of repeated domains of about forty amino acids in length (Fig. 1). The distinguishing feature of these domains is the presence of a number of cysteine residues with a characteristic spacing pattern. Additional amino acid homologies can be recognized between domains in the same or different receptors, so that overall homologies between family members within these domains are in the range of 25%. These cysteine-rich domains constitute the ligand binding portion of the receptor. C-terminal to the cysteine-rich domains are stretches of amino acids, of variable length, that are rich in proline, serine and threonine residues and are therefore predicted to be sites for addition of O-linked glycosylation. CD30 is thus far unusual in that it has a partial duplication of the cysteine-rich and O-linked sugar-rich regions. The cytoplasmic domains of the receptors are of variable length and show no homology to other proteins of known function, such as tyrosine kinases. While there is no general sequence motif common to all family members, some limited sequence homology has been recognized between CD40, TNFRp55 and Fas. A so-called "death" domain, responsible for the triggering of apoptotic cell death, has been defined in the Fas cytoplasmic domain, and this region shows homology to TNFRp55 [18, 19]. The cytoplasmic domains of 4-1BB and CD27 also share a region of sequence homology [20]. Clearly, the strong sequence homology between the receptor family members suggests that the genes evolved from a common ancestor by duplication and divergence. This is supported by similarities in

Table I. Chromosomal locations of the genes for the TNF-related ligands and their receptors

	Human	Mouse
Receptors		
TNFRp75	1 p36	4 distal
CD30	1 p36	
4-1BB		4 distal
OX40		
TNFRp55	12 p13	6
TNFR-RP	12 p13	
CD27	12 p13	
CD40	20	2 distal
Fas	10 q24	19 distal
Ligands		
TNF-α	6 (MHC)[1]	17 (MHC)
LT-α	6 (MHC)	17 (MHC)
LT-β	6 (MHC)	17 (MHC)
CD27L	19 p13	
4-1BBL	19 p13	17 central
CD30L	9 q32	4 central
CD40L	X q26	X proximal
FasL		1 distal

[1]MHC refers to a location within the major histocompatibility complex for the closely linked TNF-α, LT-α and LT-β genes. The information is compiled from the following sources: TNFRp75 [115, 116], CD30 [117], 4-1BB [26], TNFRp55 [115, 116], TNFR-RP [17], CD27 [22], CD40 [118, 119], Fas [105, 120], LT-β [28], CD27L [25], 4-1BBL [26], CD30L [24], CD40L [60], FasL [121].

intron/exon organization as well as close genetic linkage for the TNFRp55 , CD27 and TNFR-RP genes [17, 21, 22] (Table I).

The Ligand Family

The functional activity of cross-linked antibodies to various members of the TNFR/NGFR family strongly indicated that natural ligands with cytokine-like activity would exist for these orphan receptors. Indeed, in the past two years, ligands for CD40, CD30, CD27, 4-1BB and Fas have been identified and cloned [23-27]. All have proved to be type II membrane glycoproteins with homology to TNF within their C-terminal extracellular domains (Fig. 2). Additionally, another member of the family, lymphotoxin-β (LT-β), was identified as a cell-surface protein complexed to TNF-β (also known as lymphotoxin-α and referred to here as LT-α. The term TNF will be used when describing properties shared by TNF-α and LT-α) [28].

Structures of the Ligands and Ligand: Receptor Complexes

TNF-α is found as both a cell-surface, type II membrane glycoprotein and as a proteolytically cleaved, soluble form. LT-α is a secreted protein but can also be found complexed to LT-β on the membrane (discussed below). The structures of TNF-α and LT-α have been determined by X-ray crystallography [29-31]. Both molecules are trimeric, and each monomer is composed primarily of β-strands. Two sheets formed by eight antiparallel β-strands are arranged in a sandwich structure, described as a β-jellyroll. Alignment of the carboxy-terminal sequences of the ligand family members (~150 amino acids) with TNF-α and LT-α shows amino acid identities ranging from 12-29%, with particular conservation within the β-strand regions, including the residues in TNF-α and LT-α that are involved in intersubunit contacts. Indeed, it is possible to model the structure of CD40L on those of TNF-α and LT-α and achieve good superimposition of the β-strands [32]. This type of analysis strongly suggests that all the TNF-related ligands will share the same β sandwich structure and will likely be multimers, perhaps trimers like TNF-α and LT-α.

A major advance in the understanding of how these ligands bind to their receptors and trigger signaling came with the determination of the crystallographic structure of LT-α bound to the extracellular domain of the TNFRp55 [33]. The LT-α trimer can be visualized as a pyramid-like structure with three vertical surface grooves formed at the intersubunit interfaces. Each receptor monomer forms an elongated structure, slightly bent, with the four cysteine-rich domains stacked on top of each other. Each receptor monomer binds to the interface between LT-α monomers and thus makes contact with two ligand monomers. Conversely, each LT-α monomer binds two receptors. However, there is no contact between receptors in the ligand binding region. These interactions are shown schematically in Figure 3. This receptor-ligand structure immediately suggests that receptor binding to ligand will induce clustering of receptors within the transmembrane and

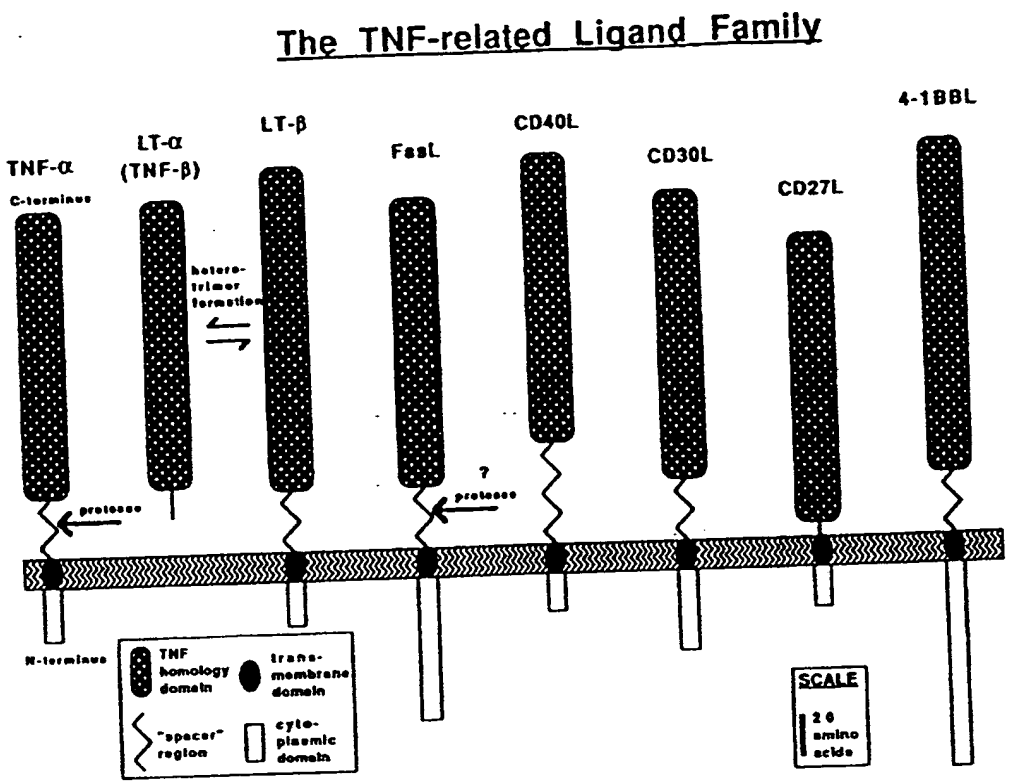

Fig. 2. Schematic representation of the structures of the TNF-related ligand family. The indicated features of this family of type II membrane glycoproteins include a TNF homology domain of variable length and a "spacer" region. Note that LT-α (TNF-β) can be found as a secreted homotrimer or as a heterotrimer complex with membrane-bound LT-β.

cytoplasmic domains that would be the trigger for signal transduction. In other receptor families such as the tyrosine kinases and the hematopoietin receptors, receptor-receptor interactions have also been suggested to initiate signal transduction [34, 35]. This model is also consistent with the agonistic nature of many monoclonal antibodies directed against the TNFR superfamily members. The antibodies are only able to stimulate a signal when they are cross-linked, leading to receptor clustering.

CD40/CD40L

CD40 was first described as a 50 kDa glycoprotein expressed on B cells and B cell precursors as well as epithelial cells [36]. Cross-linked antibodies to CD40 were shown to mediate profound effects on B cells, including homotypic adhesion via induction of type one intracellular adhesion molecule (ICAM-1) and CD23, elevation of B7 and class II major histocompatibility (MHC) antigen expression, costimulation of proliferation in conjunction with anti-IgM or phorbol myristate acetate (PMA) or interleukin (IL)-4, and costimulation of immunoglobulin (Ig) secretion with IL-4 [37-42]. Of particular interest was the observation that human B cells could be grown in long-term culture when stimulated by anti-CD40 presented by L cells expressing Fc receptors in the presence of IL-4 or IL-10 [43, 44]. The choice of cytokine greatly influenced the pattern of Ig isotypes produced in these cultures. Specifically, IL-10 caused upregulation of IgM, IgG1, IgG2,

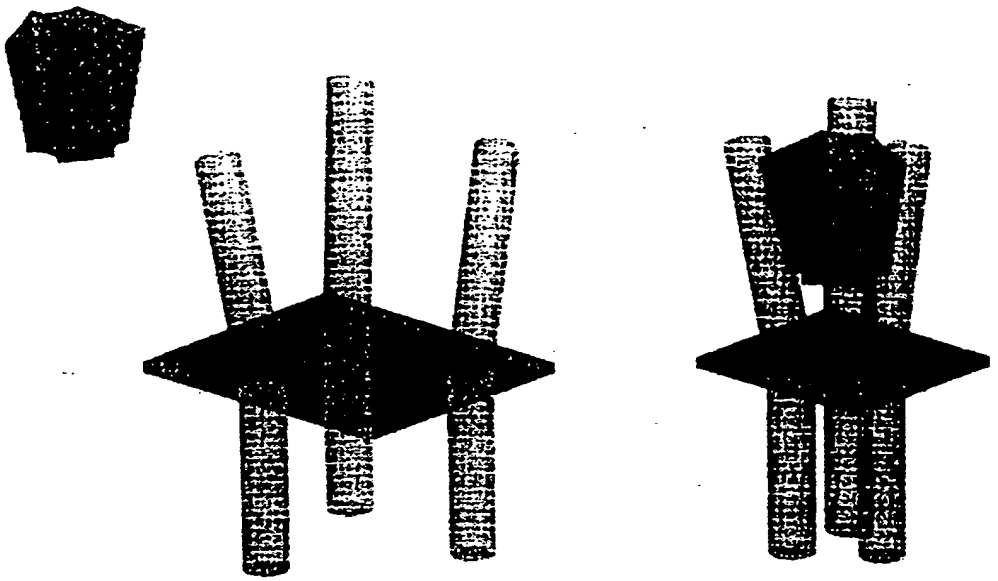

Fig. 3. Schematic representation of an LT-α (TNF-β) homotrimer binding to its receptor. As discussed in the text, each receptor monomer (shown as a rod) binds to the interface between ligand monomers (shown as a groove in the pyramid-like structure). Binding of the ligand to the three receptor monomers causes clustering, bringing together the cytoplasmic domains of the receptor and initiating signal transduction.

IgG3 and IgA, while IL-4 stimulated IgG4 and IgE production [45, 46]. Antibodies to CD40 have also been shown to prevent apoptosis of germinal center centrocytes in culture [47]. CD40 itself was cloned in 1989 [11] and shown to have the structure depicted in Figure 1.

In order to search for a source of CD40L, a chimeric fusion protein was produced that joined the extracellular domain of CD40 to the Fc region of human IgG1 [48]. This dimeric molecule could then be used as a surrogate antibody to detect CD40L expression using flow cytometry. The mouse thymoma line, EL-4, was found to show specific binding of CD40.Fc and subjected to fluorescence activated cell sorting to derive a cell population expressing higher levels of the putative ligand. The ligand was then cloned by direct expression in mammalian cells using radiolabeled CD40.Fc binding to detect transfected cells expressing the ligand [23]. The murine CD40L sequence was then used to clone the human equivalent by cross-species hybridization or polymerase chain reaction (PCR) [49, 50]. The structure of the ligand is shown in Figure 2: the extracellular, C-terminal domain contains four cysteine residues in the mouse (five in the human) and one N-linked glycosylation site. The human and murine amino acid sequences are 78% identical. Its homology to TNF has already been discussed, and of all the currently known TNF-related ligands, CD40L is distinguished by having the longest "spacer" between the transmembrane domain and the TNF homology region.

The recombinant CD40L, either expressed as a cell-surface protein on transfected cells or as a soluble molecule (rendered trimeric by fusion to either the extracellular domain of CD8 or a modified leucine zipper motif), possesses all the biological activities on B cells predicted from studies using anti-CD40 antibodies [23, 49-55].

The CD40L is expressed predominately on activated $CD4^+$ T cells, but also on a small proportion of $CD8^+$ T cells [49]. It is also expressed on mast cells and basophils [56]. Both natural and recombinant CD40L are visualized as a single band of 33 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) under reducing or non-reducing conditions [23]. As discussed above, the multimeric

status of CD40L is unknown, although modeling studies would suggest a trimer.

It had been known for some time that activated helper T cells could provide a contact-dependent signal to B cells in vitro that stimulated proliferation and, if cytokines were present, Ig production [57]. This activity could be demonstrated with fixed T cells or with membrane fractions. Blocking studies with CD40.Fc or with a monoclonal antibody, subsequently shown to recognize CD40L, demonstrated that CD40L was the molecule responsible for this activity [58, 59].

Despite the multitude of examples of CD40L activities on B cells in vitro, the proof of the importance of CD40/CD40L in vivo came from the demonstration that mutations in the CD40L gene were the cause of a human immunodeficiency disease. The mapping of the CD40L gene to Xq26 immediately suggested that hyper IgM syndrome (HIGM), previously mapped to this locus, might be a result of disruption of CD40L function [60, 61]. Males with this condition suffer from recurrent bacterial infections starting from birth. Although they have normal numbers of circulating B cells and normal to greatly elevated levels of IgM and IgD, they have no germinal centers and low or absent IgG, A and E [62]. Activated T cells from HIGM patients were unable to bind CD40.Fc, although some could bind a polyclonal antibody to CD40L [60, 63-66]. Sequencing of CD40L cDNA from the HIGM patients revealed a variety of point mutations, which, when introduced into a CD40L expression vector, abrogated all tested in vitro functional assays of the ligand [60, 63]. The mutations, both single amino acid substitutions and deletions, mapped to the TNF homology region of the ligand. One exception introduced a charged amino acid into the hydrophobic transmembrane domain, thus preventing cell surface expression of CD40L [60, 63, 64, 66]. Finally, it was shown that B cells from HIGM patients could respond normally in vitro, when stimulated with wild type CD40L, in proliferation and Ig secretion assays [60, 63]. These studies demonstrate conclusively that CD40L is required for isotype switching and affinity maturation in the germinal center and prove the importance of T cell-expressed CD40L for B cell differentiation. Although CD40L can be shown to stimulate both B cell proliferation and IgM production in vitro, it may be that in vivo the recurrent bacterial infections stimulate other pathways of B cell activation and IgM production.

Some other clinical features of HIGM patients are not so easily explained by absent CD40L stimulation of B cell function. In particular, neutropenia and infection by intracellular pathogens such as *Pneumocystis carinii* are common [62]. Perhaps CD40L has functions on other cell types? In this regard, an increased number of such functions have recently been demonstrated. CD40L can stimulate the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) from thymic epithelial cells in the presence of IL-1 and interferon (IFN)-γ [67]. CD40 was discovered to be expressed at low levels on monocytes and greatly upregulated by IL-3, GM-CSF, and IFN-γ [68]. Recombinant CD40L-mediated triggering of these cells leads to IL-6 and IL-8 production which is markedly enhanced by IL-3 or GM-CSF. IFN-γ can enhance IL-6 but not IL-8 production. Although CD40L alone did not induce TNF secretion, CD40L synergized strongly with IL-3, GM-CSF or IFN-γ to stimulate the production of TNF by monocytes. Finally, CD40L alone induced strong tumoricidal activity of monocytes against a melanoma target. T cells, too, express low levels of CD40, and the activities of recombinant CD40L so far reported on T cells are upregulation of IL-2Rα (CD25), CD69, and CD40L itself; stimulation of IL-2, IFN-γ and TNF-α production; and costimulation of proliferation in conjunction with a variety of agents such as phytohemagglutinin (PHA) or anti-CD3 [69]. Although CD40L does induce low levels of IL-2 production by T cells, the proliferation mediated by CD40L costimulation on both $CD4^+$ and $CD8^+$ T cells is largely IL-2 independent.

While it is not yet possible to prove directly that these non-B cell activities of CD40L are directly responsible for the neutropenia and intracellular infections seen in HIGM patients, the subject merits more study. Already there is enough evidence to support a role for CD40L in immune activation in which it participates not only in the stimulation of B cell differentiation towards affinity maturation and isotype switching, but also the enhancement of the function of the B cell as an antigen presenting cell (via upregulation of class II MHC and the costimulatory molecule B7), the activation and expansion of T cells, and the stimulation of cytokine secretion and functional activation of monocytes.

A critical role for CD40L in immune activation is also supported by the finding that an

antibody to CD40L can block both primary and secondary humoral immune responses to T-dependent antigens in vivo [70]. This antibody was also able to prevent collagen-induced arthritis, a mouse model of autoimmune disease [71].

CD27/CD27L

CD27 is a 55 kDa, disulfide-linked, homodimeric glycoprotein whose expression has so far been described only on lymphocytes [72]. Cloning of CD27 revealed it to be a member of the TNFR family [13]. Both agonistic and antagonistic monoclonal antibodies to CD27 have been reported. The former are able to costimulate T cell proliferation in conjunction with PHA or anti-CD3 [72], while the latter have been reported to inhibit T cell proliferation induced by various stimuli, including antigen, anti-CD2, anti-CD3 or PHA [73]. One report describes inhibition of pokeweed mitogen-induced B cell IgG synthesis by anti-CD27 [73]. CD27 expression is induced upon T cell activation [74], and a soluble form of the molecule, comprising the extracellular domain, is shed by proteolytic cleavage [75]. Increased circulating levels of soluble CD27 are a marker for T cell activation [76]. CD27 is expressed on both $CD45RA^+$ naive T cells and $CD45RO^+$ primed T cells [77]; however, CD27 expression is absent from a subpopulation of $CD45RO^+$ T cells that has been shown to include fully differentiated, mature effector T cells [78]. CD27 expression cannot be reinduced in these cells.

On B cells, CD27 expression is only found on cells that have differentiated sufficiently to be able to secrete Ig [79]. It is not expressed by naive B cells. CD27 is also expressed on some natural killer (NK) cells and upregulated by IL-2. Antagonistic anti-CD27 antibodies can inhibit IL-2 induced NK cytolytic activity [80].

The CD27L was identified and cloned using a CD27.Fc fusion molecule largely as described for CD40L [25]. Relatively high levels of CD27.Fc binding were found on an Epstein-Barr virus (EBV)-transformed, lymphoblastoid cell line, MP-1, and this was used as the source for expression cloning of CD27L. The CD27L cDNA showed a typical sequence for a member of the TNF-related ligand family, with four cysteine residues and two N-linked glycosylation sites in the extracellular domain. The recombinant protein showed an apparent molecular weight of 29 kDa or 50 kDa under reducing conditions when expressed in different cell types [25, 81]. The CD27L mRNA is detectable in cells of T, B and monocytic lineages, and the protein is upregulated upon T or B cell activation. Very recently, CD27L was shown to be identical to CD70 [82]. The recombinant CD27L is able to costimulate T cell proliferation as expected by studies with anti-CD27 [25]. This proliferation is independent of IL-2. CD27L also enhances Cytotoxic T Lymphocyte (CTL) generation in a lectin-mediated killing assay [25]. The ability of an antibody to CD27L to block allogeneic B cell-induced stimulation of T cell proliferation suggests that certain antigen presenting cells, like B cells, may use CD27L as an important second signal for T cell recruitment and expansion [81]. While a conceptual framework is emerging for the function of CD27/CD27L on T cells, much more study is needed of its role on B cells and NK cells.

4-1BB/4-1BBL

4-1BB was identified as a murine cDNA whose corresponding mRNA was upregulated upon T cell activation [14]. It was subsequently recognized as a member of the TNFR superfamily [6], but little else was known about the protein and its ligand until recently. Generation of an antibody to 4-1BB allowed its detection as a 30 kDa glycoprotein found in monomeric and dimeric forms on activated T cells and thymocytes [83]. As might be predicted, the antibody was costimulatory for T cell proliferation. The cytoplasmic domain of 4-1BB contains a consensus site for Lck binding as is found in CD4 and CD8, and 4-1BB can be shown to bind Lck [84]. Although one report shows that murine 4-1BB can bind to extracellular matrix proteins, it is not clear that this has any functional significance [85].

The murine 4-1BB ligand was identified and expression cloned using a 4-1BB.Fc fusion protein and EL-4 cells as a source [26]. Unlike the other members of the TNF-related ligand family which show sufficient homology to allow cross-hybridization between human and mouse cDNA (between 70 and 80% amino acid identity), the human 4-1BBL proved impossible to detect using this method. Instead, a human 4-1BB cDNA was obtained using the murine probe that had 60%

amino acid identity. A human 4-1BB.Fc fusion protein was then constructed and used to expression clone 4-1BBL from an activated human T cell clone [86]. The human and murine ligand sequences shared only 36% amino acid identity. The genes for both human and murine 4-1BB ligands are closely linked to the *vav* gene, suggesting that they really are homologs and not two distinct ligands for 4-1BB. The 4-1BBL is the largest of the TNF-related ligand family and is found as a disulfide linked, glycosylated homodimer of 100 kDa. The expression of the 4-1BBL, at least at the message level, appears quite broad. Transcripts can be found not only in activated T cells but in bone marrow and thymic stromal cells, activated macrophages, EBV-transformed B cells, a neuroblastoma cell line, a megakaryoblastic leukemia cell line and a variety of tissues. However, the only biological activity of the ligand that has been described is costimulation of T cell and thymocyte proliferation. Clearly, much remains to be learned about this ligand-receptor pair.

OX40

OX40 is one of the least well characterized of the TNFR family. It was recognized in the rat by the generation of a monoclonal antibody, specific for activated T cells, that could costimulate T cell proliferation [87]. Both rat and mouse OX40 cDNA were subsequently cloned and used to confirm the restricted expression of the protein [16, 88]. Cloning of the OX40 ligand has not yet been reported, although one paper reports the staining of a subset of B cells with an OX40.Fc fusion protein, and the precipitation of a 70 kDa cell-surface protein using this reagent [88]. It remains to be determined if this represents a *bona fide* OX40 ligand.

Lymphotoxin-β (LT-β)

LT-β was detected as a membrane protein, expressed on activated T cells, B cells and NK cells, complexed with LT-α [89]. LT-α is more commonly found in a soluble homotrimeric form, so it was assumed that the cell surface LT-α:LT-β complex was a heterotrimer of both 1:2 and 2:1 stoichiometry, after the cloning of LT-β revealed it to be a type II membrane glycoprotein belonging to the TNF-related ligand family [28]. Interestingly, the LT-β gene is very closely linked to the TNF-α and LT-α genes in the MHC region. Very recently it was determined that the TNFR-RP may be a receptor for LT-β [90]. The recombinant TNFR-RP, expressed as an Fc fusion protein, precipitated LT-β alone (presumably a homotrimer) or a heterotrimer of two molecules of LT-β and one molecule of LT-α. However, TNFR-RP did not bind LT-α alone or a heterotrimer of two molecules of LT-α and one molecule of LT-β. Conversely, TNFRp55.Fc bound LT-α homotrimers and the 2:1 LT-α:LT-β complex but not LT-β homotrimers or the 2:1 LT-β:LT-α complex. It is not clear whether the heteromeric complexes would be expected to signal through TNFR or TNFR-RP because only a maximum of two receptors of one type could bind the complex. (As discussed above, each receptor monomer binds to the cleft between two ligand monomers and would be specific for a cleft between identical monomers.) Neither has it yet been demonstrated that LT-β homotrimers can signal through TNFR-RP, although it seems likely. The ability of TNF-related ligands to form heteromeric complexes, although yet to be demonstrated with any other family members, adds greatly to the potential biological complexities of the systems. It also raises the possibility of heteromeric receptor complexes.

CD30/CD30L

CD30 is unique among the TNFR family members in that it has been extensively studied for more than a decade as a clinical marker for various lymphomas [91]. Despite this, we understand very little of its biological role. High levels of CD30 are usually expressed on the Reed-Stemberg cells of Hodgkin's disease and on a subset of non-Hodgkin's lymphoma known as large cell anaplastic lymphoma (LCAL) [92]. It is also found on a proportion of other lymphomas such as Burkitt's and adult T cell leukemia/lymphoma, as well as a variety of tumors of epithelial and mesenchymal origin [92, 93]. On normal cell populations, CD30 is expressed on subsets of activated T and B cells, and one report describes expression on monocytes differentiated in vitro [94].

CD30 is a 120 kDa cell-surface glycoprotein [95] but has also been reported to exist as an independently synthesized intracellular 57 kDa

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protein [96]. The molecular cloning of CD30 showed it to have an unusual structure for a TNFR family member, with a duplication of cysteine rich and serine-threonine rich domains (Fig. 1) [12]. The structure of the cDNA did not reveal how a 57 kDa intracellular form could be synthesized.

Once again, a CD30.Fc fusion protein was used to identify and clone a CD30 ligand cDNA from an activated murine T helper cell clone [24]. The murine cDNA was then used to isolate the human homolog from an activated T cell cDNA library. The CD30 ligands have a typical structure for members of the TNF related family. There are five (human) and six (mouse) N-linked glycosylation sites in the extracellular domain and five cysteine residues. Mouse and human sequences share 72% amino acid identity. The protein is detected as a 40 kDa monomer on SDS gels under reducing conditions that forms disulfide linked dimers and higher multimers in the absence of reduction. It is expressed by activated T cells and monocytes.

The recombinant CD30 ligand and cross-linked monoclonal antibodies to CD30 can costimulate T cell proliferation as is found with other family members. This is accompanied by an upregulation of ICAM-1 expression by the T cells. The ligand can also enhance the proliferation of several Hodgkin's disease derived Reed-Sternberg cell lines, suggesting the possibility that an interaction between CD30L on activated T cells, surrounding Reed-Sternberg cells in a malignant lymph node, and CD30 on the Reed-Sternberg cells might contribute to the disease process. In contrast, recombinant CD30L mediated a cytostatic and cytotoxic effect on LCAL-derived cell lines in culture, suggesting a possible clinical utility for the ligand or agonistic anti-CD30 antibodies [6, 97].

Fas/FasL

The Fas/APO-1 antigen was discovered by the generation of monoclonal antibodies selected for their ability to kill cell lines in vitro [98, 99]. It was established that the killing was via induction of apoptosis (active or programmed cell death) in the target cell. The antibodies were used to show that Fas is a 50 kDa membrane glycoprotein, to clone the Fas cDNA and to identify Fas as a TNFR family member [15]. Because TNF is also able to mediate apoptosis in some cell lines, comparisons were made between the cytoplasmic domains of Fas and the TNFRs. A region of 65 amino acids showing 28% amino acid identity between Fas and TNFRp55 was identified and shown by mutational analysis to mediate cytotoxicity in both molecules [18, 19]. The Fas protein was also shown to contain a short stretch of amino acids at its C-terminus that negatively regulated cytotoxicity [18]. Deletion of the C-terminal 15 amino acids enhanced Fas-mediated killing.

The Fas antigen has quite a wide distribution. It is found on thymocytes, CD45RO$^+$ (primed or memory) T cells, B cells, and monocytes as well as liver, heart, lung and ovary [100, 101]. Many cultured cell lines of diverse origin express Fas. Of particular note is that Fas-mediated cell death is usually observed only with cultured cell lines and not with primary cell cultures. (The exception is the hepatotoxicity caused by injection of anti-Fas antibody [102].) Freshly isolated T cells, for example, which express Fas, are resistant to Fas-mediated killing, but acquire sensitivity after several days in culture [103]. Indeed, it has recently been shown that Fas antibodies can costimulate the proliferation of freshly isolated thymocytes or T cells [104]. In this respect, Fas antibodies resemble antibodies or ligands for the other TNFR family members, and the parallel with the TNF system is especially close as TNF has been shown to costimulate both T and B cell proliferation as well as to mediate apoptosis of certain cell lines.

An extremely important advance in our understanding of Fas biology came with the finding that the genetic lesion of the *lpr/lpr* mouse, a model for spontaneous autoimmune disease, resides in the Fas gene [105]. The *lpr* (cg) allele carries a mutation in the cytoplasmic domain of Fas that was shown to abolish Fas-mediated cytotoxicity, and the other known *lpr* allele expresses little if any functional Fas mRNA due to the insertion of a transposable element into an intron of the Fas gene [105, 106]. Thus, cells from *lpr/lpr* mice are resistant to Fas antibody-mediated killing. What is the connection between Fas defects and autoimmune disease? The first suggestion was that Fas was involved in negative selection of potentially autoreactive T cells during their development in the thymus. However, a test of this hypothesis showed that negative selection

appeared intact in *lpr/lpr* mice [107]. An alternative hypothesis views the conversion from a Fas-mediated activation signal to a Fas-mediated cytotoxic signal as being important in regulating self-reactive lymphocytes in the periphery as well as a mechanism for downregulating an immune response once the antigen has been cleared from the system [104, 108]. Loss of Fas activity would lead to the accumulation of lymphocytes that would normally have undergone apoptosis, some of which would be autoreactive.

The Fas ligand has very recently been cloned. A key finding was that the Ca^{2+}-independent cytotoxicity of a rat-mouse T cell hybridoma, selected for its high levels of cytotoxicity, was mediated by Fas ligand:Fas interactions [109]. This cytotoxicity was rapidly induced by PMA and ionomycin treatment. Cells from *lpr/lpr* mice were resistant to this killing, and susceptibility to Ca^{2+}-independent killing could be conferred to a resistant cell line by transfection of Fas. Similar data were obtained using PMA and ionomycin activated, antigen-specific, normal peritoneal exudate lymphocytes as the cytotoxic cells. The rat-mouse hybridoma was then shown to bind a Fas.Fc fusion protein, sorted for increased Fas ligand expression, and used to construct an expression cDNA library from which the ligand cDNA was isolated and shown to mediate apoptosis by triggering Fas [27]. The FasL, which turned out to be of rat origin, shows a typical TNF-related ligand structure and indeed resembles TNF more than other family members. Like TNF, but unlike other family members, a biologically active, soluble form of the molecule is generated at relatively high levels upon transient expression in COS cells in addition to the membrane bound form. Whether a soluble form of FasL occurs naturally remains to be determined. FasL mRNA can be detected in testis, small intestine, lung and kidney, as well as in activated thymocytes and splenocytes. Very recently the FasL has been identified as the product of the mouse *gld* gene [110]. This had been previously expected for several reasons. Mice homozygous for the *gld* mutation have an identical phenotype to *lpr/lpr* mice, but their cells express Fas and can be killed by anti-Fas antibodies. Antigen-specific peritoneal exudate lymphocytes from *gld/gld* animals cannot mediate Ca^{2+}-independent cytotoxicity, whereas *lpr/lpr*-derived cells can [109], and *gld/gld*-derived activated T cells cannot bind Fas.Fc, whereas equivalent cells from *lpr/lpr* mice can [111].

Conclusion

The discovery of the families of molecules related to TNF and its receptors has opened an important new area of research with major insights having already been obtained in the understanding of the control of immune activation and differentiation. The existence of spontaneously occurring mutations in ligand or receptor genes (*lpr/lpr* mice and HIGM patients) has been particularly helpful in this regard. The creation of mice in which the TNFRp55 gene is disrupted has also shown the importance of this receptor in the response to infection by certain pathogens [112, 113]. In view of the pleiotropic and redundant biological activities of the recombinant ligands in vitro, the construction of more such "knock-out" mice will be essential in understanding the role of each family member.

Apart from the structural similarities and the shared feature of multimeric ligands signaling through receptor clustering, other common elements of this family of molecules are ligand-induction of cytokine expression, upregulation of adhesion molecules and activation antigens, the shedding of soluble receptors after activation, and stimulation of cell proliferation and cell death on different populations. Table II shows our current state of knowledge on the expression of the ligands and receptors on cells of the immune system. It is immediately obvious that all the receptors and ligands are expressed on activated T cells. As mentioned above, all the ligands share the property of costimulation of T cell proliferation, which would suggest redundancy of function. However, even the limited studies that have been done to date suggest that the receptors and ligands will be found on different lymphocyte or monocyte subpopulations, will have different requirements for induction, and will be induced and expressed with different kinetics. There is also a good possibility that synergistic interactions will take place between different ligands. As has been found with other receptor families, it is likely that the TNFR-related receptors will activate more than one intracellular signaling pathway. Some of these pathways will be common to several receptors, others will be unique. Recently,

Table II

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Table II. Expression of TNF-related ligands and receptors by different cells of the immune system

	T cells	B cells	Monocytes	Other
CD40L	+ (act.)[1]	–[2]	–	mast cells basophils
CD40	+	+[3]	+ (act.)	thymic epithelial cells
CD30L	+ (act.)	–	+ (act.)	
CD30	+ (act.)	±[4] (act.)	±	
CD27L	+ (act.)	+ (act.)	±	
CD27	+	±	–	
4-1BBL	+ (act.)	±	+	
4-1BB	+ (act.)	–	–	
FasL	+ (act.)	–	–	
Fas	+	+	+	

[1] + (act.) means the expression of the protein is induced or increased upon activation of the cell.
[2] – indicates that expression of the molecule has not yet been convincingly demonstrated.
[3] + indicates the molecule is constitutively expressed.
[4] ± used either where conflicting reports exist, or where expression has been detected in cell lines of a given lineage, but not on primary cells.

another facet of the biology of this ligand-receptor family has begun to emerge: the reciprocal transmission of a signal through the ligand as well as through the receptor following ligand-receptor interaction. This has now been documented for both CD40L and CD27L (CD70) [82, 114]. Understanding these issues will be a challenge for years to come. Finally, although most work on this family has focused on the immune system, at least some of the receptors have a much broader distribution (TNFR, Fas, CD40), implying other, as yet unknown functions. This area, too, is worthy of more attention.

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